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Cyclohexylamine inhibits the adhesion of lymphocytic cells to human syncytiotrophoblast

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Abstract

We have previously shown that lymphocytic cells adhere to cultured syncytiotrophoblast and that this may be important in the lymphocyte-mediated infection of trophoblast with the human immunodeficiency virus (HIV). During the course of studies aimed at investigating the role of cell surface carbohydrates in adhesion, it was discovered that a contaminant of commercial fucose-1-phosphate, dicyclohexylamine, inhibited MOLT-trophoblast adhesion. Dicyclohexylamine and the related compounds, cyclohexylamine and hexylamine, inhibited adhesion in a dose-responsive manner with half-maximal inhibition seen at about 4 mM. While the pressor effects of cyclohexylamine, the principal metabolite of cyclamate, are well known, this is the first report of an effect of this and related compounds on cell adhesion activity. The inhibitory effect was reversible and, at concentrations less than 25 mM, did not result in loss of cell viability. Several possible mechanisms of action of cyclohexylamine were examined in an attempt to explain the effect on adhesion. No evidence was found to suggest that the effects of cyclohexylamine were due to inhibition of polyamine synthesis, increase in intracellular Ca^{2+} concentration or to a lysosomotropic effect. The concentrations of cyclohexylamine used are within the range of plasma concentrations attainable in humans, raising the possibility that the in vitro effects described here may also occur in vivo. The results also suggest that caution should be used in the interpretation of results obtained from experiments where cell adhesion is blocked using exogenous monosaccharides that are in the form of dicyclohexylammonium salts. Appropriate controls must be included or, if possible, sodium, potassium or barium salts should be chosen.

Keywords: Trophoblast; Cell adhesion; Cyclohexylamine; Adhesion molecule; Leukocyte; Fucose-1-phosphate

1. Introduction

We have previously demonstrated that lymphocytic cells adhere to human syncytiotrophoblast cultures in a time-dependent manner [1]. These experiments also showed that adhesion was Ca^{2+}- and Mg^{2+}-dependent and temperature sensitive. We recently reported that adhesion was partly mediated through lymphocyte VLA-4 ($\alpha 4$ integrin) and an as yet unidentified counter-receptor on trophoblast [2]. The adhesion of lymphocytes to syncytiotrophoblast may have important physiological and immunological consequences and may be an important event in the lymphocyte-mediated infection of trophoblast with HIV [1,3].

Many adhesion molecules are glycoproteins and in several instances adhesion requires the expression of specific cell-surface carbohydrate moieties that serve as ligands for adhesion molecules. Much information about the nature of the carbohydrates that are involved has come from studies that examine the blocking effects of exogenous carbohydrates on cell–cell adhesion [4–6]. Most commercially available phosphorylated monosaccharides that are used as blocking reagents are offered as sodium, barium or potassium salts. Some, however, are also available, or are only available, as dicyclohexylammonium salts. During the course of studies aimed at characterizing the role of cell surface carbohydrates in the attachment of lymphocytes to human trophoblast cells, it was fortuitously discovered that adhesion could be inhibited by dicyclohexylamine and, subsequently, by cyclohexylamine. These findings are reported and discussed here. The implications of these results for the interpretation of carbohydrate blocking experiments are also discussed.

Cyclohexylamine is the principal metabolite of the arti-

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ficial sweetener, cyclamate [7]. It is well established that cyclohexylamine acts as a sympathomimetic amine causing the release of noradrenaline and so indirectly increasing blood pressure in both laboratory animals and in humans [8–11]. Cyclohexylamine is also reported to cause testicular atrophy in rats [12–14]. Other less well known effects of cyclohexylamine include its ability to inhibit polyamine synthesis [15,16] and to act synergistically with phorbol ester to inhibit metabolic cooperation [17]. To our knowledge, there have been no previous reports of the effects of cyclohexylamine on cell adhesion events.

2. Materials and methods

2.1. Materials

Calcein-AM was obtained from Molecular Probes, Eugene, OR, and stored as a 1 mM stock solution in DMSO at −70° C. Fura-2-AM was also obtained from Molecular Probes and stored as a 3.7 mM solution in DMSO at −70° C. Human recombinant tumor necrosis factor-α (TNF-α; $2 \cdot 10^7$ units/mg) was purchased from Genzyme Corporation, Cambridge, MA. Phosphorylated monosaccharides, neutral monosaccharides, fucoidan, mannan, chondroitin sulfate and dextran sulfate were obtained from Sigma Chemicals, St. Louis, MO. Phosphorylated monosaccharides were obtained as barium, sodium or dicyclohexylammonium salts as indicated in the text. Swainsonine, 1-deoxynojirimycin, castonospermine and 1-deoxymannojirimycin were also purchased from Sigma. Neuraminidases from *Vibrio cholerae* and *Arhrobacter ureafaciens* were also obtained from Sigma. Dicyclohexylamine, cyclohexylamine phosphate and hexylamine were obtained from Sigma. Chloride forms of dicyclohexylamine and hexylamine were prepared by reaction with 11 M HCl followed by acetone precipitation. Spermine and spermidine were obtained from Sigma. Monensin was obtained from Calbiochem, San Diego, CA and stored as a 1 mM stock solution in ethanol at 4° C. The pH of all solutions was routinely checked and adjusted to 7.4 if necessary.

2.2. Trophoblast isolation and primary culture

A detailed description of the procedure used to isolate and characterize cytotrophoblast cells from term human placentas has been given previously [18].

The experiments described here were performed using syncytiotrophoblast cells. The culture conditions used to induce the differentiation of cytotrophoblast to syncytiotrophoblast have been described elsewhere [19]. Cells were generally used 2 days after plating at which time more than 90% of the colonies consisted of multinucleated cells as determined by anti-desmosome/antinuclear antibody immunocytochemistry [19,20].

For cell–cell adhesion studies trophoblast cells were plated into 96-well culture dishes (Costar) and maintained in an air/CO_2 incubator at 37° C. Plating efficiency was usually about 85% and cell numbers were adjusted so that the final plating density was about 300000 cells/cm^2. Cellular protein content was determined using a modified Lowry assay containing SDS in the alkaline copper reagent [21]. Using this assay, $2.5 \cdot 10^6$ trophoblast cells were found to be equivalent to 1 mg protein.

2.3. MOLT-4 / clone 8 cells

The lymphocytic MOLT-4/clone 8 cell line was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, and maintained in RPMI-1640 containing 20% heat-inactivated fetal calf serum. Cells were used for up to 10 passages after which cultures were reestablished from the frozen stock. This was necessary because the cells began to lose their ability to bind to trophoblast after more than about 12 passages.

2.4. Cell–cell adhesion assay

A fluorescence-based assay was used in which the MOLT cells were labelled with calcein-AM and then incubated with syncytiotrophoblast cultures for 1 h at 37° C. The labelling and assay methods have been described in detail previously [1,2]. The trophoblast cultures were incubated with TNF-α (200 U/ml) for 3 h and then washed before addition of the MOLT cells [2]. Culture dishes were read in a CytoFluor 2300 fluorescence plate reader (Millipore Corporation, Marlborough, MA) using 485/22 nm excitation and 530/25 nm emission filters. Results were calculated as arbitrary fluorescence units/mg trophoblast cell protein.

2.5. Adhesion blocking experiments

For initial screening studies, calcein-AM-labelled MOLT cells were incubated with trophoblast cultures in the presence of the compound of interest. Concentrations used are given in the figure legends and text. In other experiments, either MOLT cells or trophoblast cultures were preincubated with the compound of interest for 30 min at 37° C, then washed for different periods of time before performing the adhesion assay. We also attempted to block adhesion by pretreating cells with neuraminidases from *Vibrio cholerae* and *Arthrobacter ureafaciens* [22,23] and by pretreating cultures with various inhibitors of glycoprotein processing (swainsonine, 1-deoxynojirimycin, castonospermine or 1-deoxymannojirimycin) [24].

2.6. Measurement of intracellular Ca^{2+}

Intracellular calcium levels in MOLT cells were determined using a Hitachi F-2000 spectrofluorimeter after

labelling the cells with a 7.5 μM concentration of the fluorescent calcium probe Fura2-AM [25].

2.7. Assessment of viability

For all experiments, parallel trophoblast cultures were treated with carbohydrates, inhibitors or enzymes as described above but without the addition of MOLT cells. At the end of the experiment, protein determinations were performed on these cultures to determine whether treatments caused cell detachment. Viability of both trophoblast cells and MOLT cells was also assessed by Trypan blue staining before and after cells were treated with carbohydrates or inhibitors. Because several of the compounds used in these studies had to be dissolved in either DMSO or ethanol, controls were always included to test the effects of these solvents alone. No loss of viability was observed at the solvent concentrations used for experiments.

2.8. Treatment of results

All experiments were performed at least three times and within an experiment at least triplicate measurements were made. Each experiment used cells from a different placenta. Data were analyzed using InStat software (Graph Pad Software, San Diego. CA).

3. Results

3.1. Effect of cyclohexylamine on adhesion

The effects of cyclohexylamine were only discovered after several experiments had been carried out using various commercial phosphorylated monosaccharides. For these experiments, calcein-labelled MOLT cells were incubated with trophoblast cultures in the presence of different carbohydrates and adhesion was measured. The monosaccharides used included glucose-1-phosphate, galactose-1-phosphate, mannose-1-phosphate, mannose-6-phosphate, fucose-1-phosphate, fructose-1-phosphate, lactose-1-phosphate, fucose, and mannose. Of the monosaccharides tested, only fucose-1-phosphate appeared to cause a significant dose-dependent inhibition of adhesion with a half-maximal inhibitory dose of about 3.5 mM (Fig. 1A). With the exception of fucose-1-phosphate, which was only available as a dicyclohexylammonium salt, the other monosaccharides were either sodium or barium salts. This raised the suspicion that the blocking effect might be due to dicyclohexylamine and not to the sugar itself. This was substantiated when further experiments were performed with the dicyclohexylammonium form of fructose-1-phosphate. This compound inhibited adhesion with a half-maximal inhibitory dose of 3.8 mM, whereas the sodium form had no effect (Fig. 1B,C). Unfortunately, attempts to convert the dicyclohexylammonium form of fucose-1-phosphate to a barium or sodium form using a cation exchange resin were unsuccessful. The effects of dicyclohexylamine, and of the

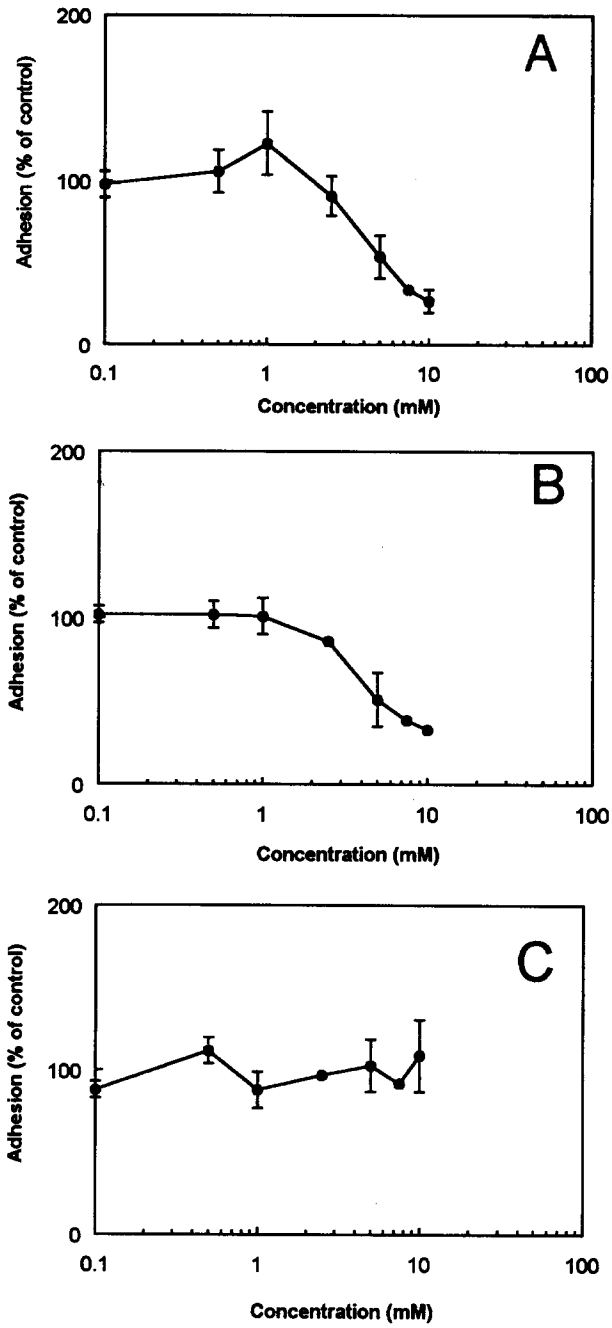

Fig. 1. Effect of different forms of fucose-1-phosphate and fructose-1-phosphate on adhesion. Calcein-labelled MOLT cells were incubated in the presence of the dicyclohexylammonium salt of fucose-1-phosphate (A), the dicyclohexylammonium salt of fructose-1-phosphate (B) or the sodium salt of fructose-1-phosphate (C) for 30 min at 37° C. The cells were then added to adherent trophoblast cultures and incubated (still in the presence of the indicated compounds) for 1 h, after which adhesion was measured as described in Section 2. Results are expressed as % of untreated control and represent mean values ±S.D. from 3 separate experiments.

related compounds cyclohexylamine and hexylamine, were then studied directly. The dose–response curves show that each of these amines inhibited the adhesion of MOLT cells to trophoblast cultures (Fig. 2A–C). Concentrations giving half maximal inhibition were 5 mM, 4 mM and 2.5 mM for dicyclohexylamine, cyclohexylamine and hexylamine, respectively. At concentrations of 10 mM and less, cyclohexylamine had no effect on trophoblast viability or attachment to the culture dish although some cytoplasmic vacuolization was seen. At concentrations of 25 mM and above, cytoplasmic vacuolization and some detachment of cells was noticed (Fig. 2).

In an attempt to determine whether cyclohexylamine was affecting MOLT cells or trophoblast or both, experiments were performed in which MOLT cells or trophoblast cells were incubated separately with cyclohexylamine and then washed before measuring adhesion. Preincubation of trophoblast cells with the amine for 30 min followed by rapid washing had no effect on adhesion, whereas preincubation of MOLT cells resulted in a 30% reduction in subsequent adhesion. The effect on MOLT cells was reversible since preincubation with cyclohexylamine followed by a long 30-min wash in fresh medium resulted in recovery of most of the adhesion activity. Identical results were obtained with dicyclohexylamine (results not shown). Thus, cyclohexylamine exerted its effect primarily on lymphocytes and not on trophoblast cells. However, it should be noted that exposure of trophoblast cultures to high concentrations of cyclohexylamine (> 10 mM) did cause cytoplasmic vacuolization and on extended exposure (> 4 h) did cause some cells to detach from the dish (results not shown).

It should be noted that other carbohydrates, including chondroitin sulfate (0.5–250 μg/ml), dextran sulfate (0.5–250 μg/ml), mannan (0.5–250 μg/ml), fucose (0.5–250 mM) and fucoidan (0.5–250 μg/ml), were without significant effect on MOLT adhesion or trophoblast attachment at all concentrations tested. Adhesion was not significantly affected by treatment of trophoblast cells or MOLT cells with neuraminidases or by preincubating trophoblast cultures or MOLT cells for 24 h with inhibitors of carbohydrate processing (swainsonine, 1-deoxynojirimycin, castonospermine or 1-deoxymannojirimycin) (results not shown).

3.2. Mechanism of action of cyclohexylamine on adhesion

The possibility that the inhibitory effect was the result of a cyclohexylamine-dependent inhibition of polyamine synthesis [16,26] was investigated. The results showed that the inhibitory effect of cyclohexylamine (5 mM) was not abrogated by either spermine or spermidine (each at 10 mM) which would have been expected if inhibition of polyamine synthesis was involved. Next, we found that intracellular Ca^{2+} levels in MOLT cells incubated with 10 mM cyclohexylamine were not significantly different from

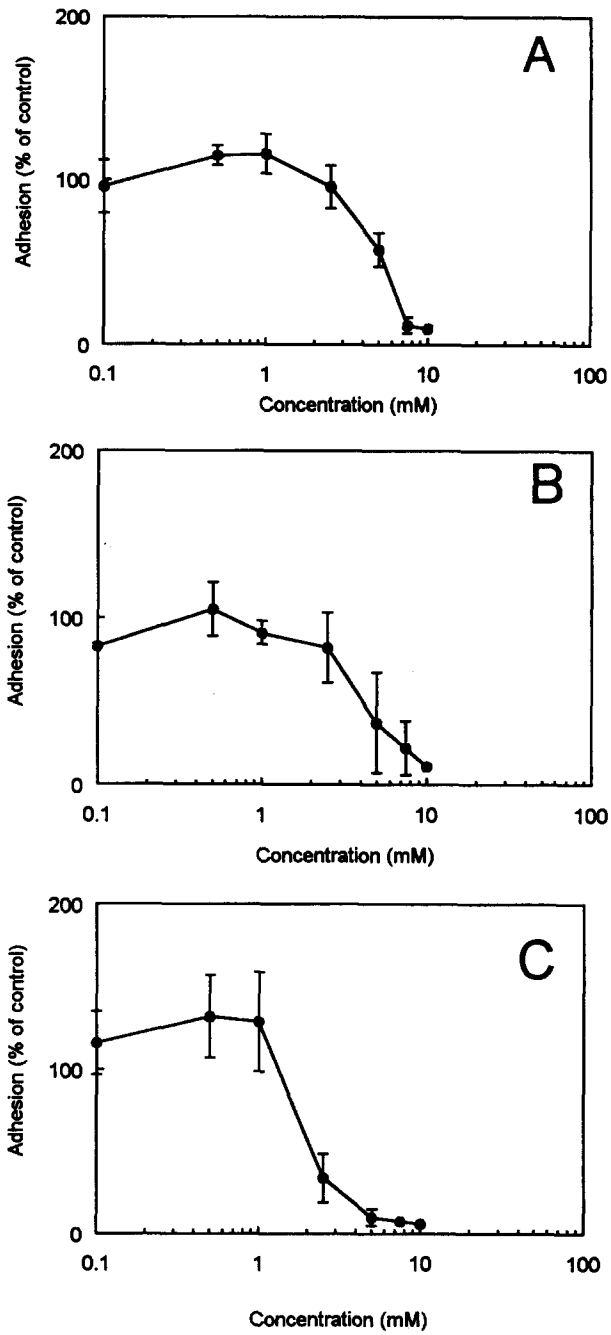

Fig. 2. Effect of different amines on adhesion. Calcein-labelled MOLT cells were incubated in the presence of dicyclohexylamine (A), cyclohexylamine (B) or hexylamine (C) for 30 min at 37° C. The cells were then added to adherent trophoblast cultures and incubated (still in the presence of the indicated compounds) for 1 h, after which adhesion was measured as described in Section 2. Results are expressed as % of untreated control and represent mean values ± S.D. from 3 separate experiments.

untreated controls, ruling out this mechanism of action [17].

The possibility that cyclohexylamine was operating as a lysosomotropic agent was tested by comparing its effects to those of the weak bases methylamine and choroquine which, like many other amines, are known to accumulate

in acidic intracellular compartments and perturb intracompartmental pH gradients [27]. Methylamine inhibited MOLT-trophoblast adhesion in a dose-dependent manner with an IC_{50} of 4 mM, while chloroquine had no effect. The ionophore monensin, which also perturbs intracellular pH gradients, but by a different mechanism, also had no significant effect on adhesion (results not shown).

4. Discussion

We have previously demonstrated that lymphocytic cells adhere to human syncytiotrophoblast cultures in a time-dependent manner [1]. These experiments also showed that adhesion was Ca^{2+}- and Mg^{2+}-dependent and temperature-sensitive. We recently reported that adhesion was partly mediated through lymphocyte VLA-4 ($\alpha 4$ integrin) and an as yet unidentified counter-receptor on trophoblast [2]. The present experiments were started with the objective of determining whether cell-surface carbohydrates played a role in the adhesion process. However, it quickly became apparent that the inhibitory effects of certain phosphorylated monosaccharides were due to the dicyclohexylamine that was present in these preparations. The other experiments shown here confirm that the related compounds, cyclohexylamine and hexylamine, also inhibit adhesion in a dose-dependent manner. To our knowledge these findings are the first to demonstrate an effect of cyclohexylamine, the major metabolite of the artificial sweetener cyclamate, on cell–cell adhesion. Previous studies have shown that cyclohexylamine inhibits polyamine synthesis [15,16] and acts synergistically with phorbol ester to inhibit metabolic cooperation [17]. The concentrations of cyclohexylamine that were effective in our studies were within the range found to be effective in these other systems and within the range of attainable plasma concentrations in humans [11]. On the basis of the in vitro results presented here, it is possible that cyclohexylamine could exert similar effects in vivo. However, the physiological consequences of such an effect remain to be explored. Cell–cell adhesion plays an important role in the control of normal leukocyte trafficking and is also important in pathological processes such as inflammation and tumor metastasis [28–30].

The mechanism by which cyclohexylamine might inhibit adhesion was also investigated. Our results indicate that inhibition of adhesion was not caused by cyclohexylamine-mediated inhibition of polyamine synthesis [15,16] or changes in intracellular Ca^{2+} levels [17] and was probably not the result of a lysosomotropic effect. In conclusion, further studies will be required to elucidate the mechanism of action of cyclohexylamine on adhesion.

The corollary of the results reported here is that the data do not provide evidence for the involvement of cell-surface carbohydrates in MOLT-trophoblast adhesion. In addition to the lack of any demonstrable effect of exogenous carbohydrates, adhesion was unaffected by pretreatment of trophoblast or MOLT cells with neuraminidase or various inhibitors of glycoprotein processing, consistent with little or no role for surface carbohydrates in this adhesion system. These findings are surprising since we have previously shown that the glycosylated integrin, VLA-4, is involved in lymphocyte-trophoblast adhesion [2]. Clearly, further studies are required to clarify the involvement of carbohydrate moieties in this adhesion system.

Finally, the inhibitory effects of dicyclohexylammonium forms of monosaccharides have important implications for others who may employ such compounds as adhesion-blocking reagents. Caution must be used in the interpretation of experiments where such compounds are used. For example, it has been reported that commercial fucose-1-phosphate reduced the binding of CD23-liposomes to RPMI-8226 cells (a B-lymphocytic cell line) and this information was used to substantiate the conclusion that a cell-surface fucose-1-phosphate moiety was involved in the adhesion reaction [31]. Since all commercially available fucose-1-phosphate is, to our knowledge, only available as a dicyclohexylammonium salt and since appropriate controls were not described, this conclusion might not be valid. Fructose-1-phosphate, mannose-1-phosphate and glucose-1-phosphate are commercially available as dicyclohexylammonium salts but, unlike fucose-1-phosphate, are also available in other forms. Clearly, sodium, potassium or barium salts should be selected where possible. If this is not possible, as is the case for fucose-1-phosphate, then controls should be included to exclude artifacts.

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References

[1] Douglas, G.C., Sloan, C.L., Hovanes, K., Thirkill, T.L., Fry, G.N., Hakim, H., Schmerl, S., Jennings, M.B. and King, B.F. (1993) J. Reprod. Immunol. 24, 65–80.
[2] Douglas, G.C., Hu, J., Thirkill, T.L., Hovanes, K., Sharma, S. and King, B.F. (1994) J. Reprod. Immunol. 24, 49–62.
[3] Douglas, G.C., Fry, G.N., Thirkill, T., Holmes, E., Hakim, H., Jennings, M. and King, B.F. (1991) AIDS Res. Hum. Retroviruses 7, 735–740.
[4] Varki, A. (1992) Curr. Opin. Cell Biol. 4, 257–266.
[5] Foxall, C., Watson, S.R., Dowbenko, D., Fennie, C., Lasky, L.A., Kiso, M., Hasegawa, A., Asa, D. and Brandley, B.K. (1992) J. Cell. Biol. 117, 895–902.
[6] Yednock, T.A. and Rosen, S.D. (1989) Adv. Immunol. 44, 313–378.

[7] Renwick, A.G. and Williams, R.T. (1972) Biochem. J. 129, 869–879.
[8] Yamamura, H.I., Lee, I.P. and Dixon, R.L. (1968) J. Pharm. Sci. 57, 1132–1134.
[9] Rosenblum, I. and Rosenblum, G. (1968) Toxicol. Appl. Pharmacol. 12, 260–264.
[10] Eichelbaum, M., Hengstmann, J.H., Rost, H.D., Brecht, T. and Dengler, H.J. (1974) Arch. Toxikol. 31, 243–263.
[11] Buss, N.E., Renwick, A.G., Donaldson, K.M. and George, C.F. (1992) Toxicol. Applied Pharmacol. 115, 199–210.
[12] Gaunt, I.F., Sharratt, M., Grasso, P., Lansdown, A.B. and Gangolli, S.D. (1974) Food Cosmet. Toxicol. 12, 609–624.
[13] Mason, P.L. and Thompson, G.R. (1977) Toxicology 8, 143–156.
[14] Creasy, D.M., Ford, G.R. and Gray, T.J.B. (1990) Exp. Mol. Pathol. 52, 155–169.
[15] Pfaller, M.A., Riley, J. and Gerarden, T. (1990) Mycopathologia 112, 27–32.
[16] Shirahata, A., Takahashi, N., Beppu, T., Hosoda, H. and Samejima, K. (1993) Biochem. Pharmacol. 45, 1897–1903.
[17] Mills, L.J., Robson, D.L. and Malcolm, A.R. (1991) Carcinogenesis 12, 1293–1299.
[18] Douglas, G.C. and King, B.F. (1989) J. Immunol. Methods 119, 259–268.
[19] Douglas, G.C. and King, B.F. (1990) J. Cell Sci. 96, 131–141.
[20] Douglas, G.C. and King, B.F. (1993) Placenta 14, 187–201.
[21] Bennett, J.P. (1982) in Techniques in Lipid and Membrane Biochemistry (Hesketh, T.R., Kornberg, H.L., Metcalfe, J.C., Northcote, D.H., Pogson, C.I. and Tipton, K.F. eds.), pp. 1–22, Elsevier, Amsterdam.
[22] Spertini, O., Luscinskas, F.W., Kansas, G.S., Munro, J.M., Griffin, J.D., Gimbrone, M.A. and Tedder, T.F. (1991) J. Immunol. 147, 2565–2573.
[23] Zhou, Q., Moore, K.L., Smith, D.F., Varki, A. and McEver, R.P. (1991) J. Cell Biol. 115, 557–564.
[24] Renkonen, R. and Ustinov, J. (1991) Eur. J. Immunol. 21, 777–781.
[25] Malgaroli, A., Milani, D., Meldolesi, J. and Pozzan, T. (1987) J. Cell Biol. 105, 2145–2155.
[26] Hibasami, H., Tanaka, M., Nagai, J. and Ikeda, T. (1980) FEBS Lett. 116, 99–101.
[27] Dean, R.T., Jessup, W. and Roberts, C.R. (1984) Biochem. J. 217, 27–40.
[28] Springer, T.A. (1990) Nature 346, 425–434.
[29] Stoolman, L.M. and Kaldjian, E. (1992) Invasion and Metastasis 12, 101–111.
[30] Albelda, S.M. (1993) Lab. Invest. 68, 4–17.
[31] Pochon, S., Graber, P., Yeager, M., Jansen, K., Bernard, A.R., Aubry, J.P. and Bonnefoy, J.Y. (1992) J. Exp. Med. 176, 389–397.